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Microplate Phosphocellulose Binding Assay for Aminoglycoside-Modifying Enzymes

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We modified the phosphocellulose binding assay for aminoglycoside-modifying enzymes (AMEs) by use of microdilution plates and a multichannel micropipette. Batteries of aminoglycoside substrates for screening organisms for the presence of AMEs as well as for subclassifying enzymes were prepared and stored in microdilution plates. When tested in parallel with the conventional tube reaction assay, the microplate assay yielded comparable radioactive counts and therefore equally correct identifications of AMEs in 32 isolates representing nine bacterial species. Other modifications, such as multichannel dispensing of crude enzyme preparations and radioisotopic precursors, provided a more rapid, convenient, and less expensive means of examining large collections of organisms for AMEs.

In pathogenic bacteria, enzymatic modification is the most common mechanism of resistance to aminoglycosides (AGs) (1). At least 27 AG-modifying enzymes (AMEs) have been described, each belonging to one of three classes: acetyltransferase (AAC), nucleotidyltransferase (ANT), or phosphotransferase (APH).

The most reliable method of identifying specific AMEs is the phosphocellulose binding assay (PBA), described in 1971 by Davies et al., which exploits the affinity of the strongly basic AGs for negatively charged media (4). If a radiolabeled moiety is enzymatically attached to a particular AG, radioactive counts on the paper will be elevated. Enzymes within any of the three major AME classes may be identified, depending upon which drugs are capable of serving as substrates. Although variations in the PBA, such as buffer composition, preparation of cell-free enzyme extracts, and other reaction parameters, have been described (3, 7, 8), the principle of the assay has not changed, and the test procedure has remained cumbersome and time-consuming. Less cumbersome means of both screening for AMEs and identifying specific enzymes are needed, especially for large collections of organisms under epidemiologic evaluation. Advances toward this end were made in 1978 by Drasar, who proposed the substitution of disposable microtiter plates for individual serologic tubes in which to perform reactions (7), and in 1982 by Shannon and Phillips, who suggested that AMEs can be screened by using only one or two AG substrates rather than the complete battery necessary for subclassifying enzymes (11). These screening methods are possible since the majority of enzymes within a class share common substrates. Gentamicin, tobramycin, or both, for example, are modified by all AACs described to date.

The purpose of our study was to develop a more convenient microdilution plate PBA for both screening for AMEs and subclassifying enzymes.

MATERIALS AND METHODS

Organisms. Five strains of *Pseudomonas aeruginosa* used as AME-positive controls were supplied by the Mechanisms of Resistance Service, Bristol Laboratories, Syracuse, N.Y. Also tested were 27 bacterial isolates (see Table 3) which represented five gram-negative species (three genera) and three *Staphylococcus* species. These isolates were obtained from hospitals and were resistant to at least one AG.

Cell-free lysates of gram-negative bacilli were prepared as follows. Brain heart infusion broth (200 ml in a 500-ml flask) was inoculated with 20 ml of an overnight broth culture and incubated with shaking at 35°C until the late exponential growth phase was reached (usually 4 to 5 h). Cells were harvested by centrifugation, washed twice in 10 ml of cold 0.01 M Tris–0.03 M NaCl (pH 7.8), and suspended in 6 ml of cold 0.3 M Tris-maleate–0.03 M $MgCl_2$–0.01 M dithiothreitol (pH 7.8). Suspensions were sonicated twice (1 min each with constant ice-ethanol cooling) with a W-375 sonic disruptor equipped with a tapered microtip probe (Heat Systems-Ultrasonics, Plainview, N.Y.), followed by centrifugation for 15 min at 12,000 × *g* and 4°C to remove cellular debris. Modifications for staphylococci included an alternative broth (Trypticase soy broth [BBL Microbiology Systems, Cockeysville, Md.] containing 0.5% glycine and 0.3% yeast extract), cellular washes in 0.01 M Tris–0.01 M $MgCl_2$–0.05 M NH_4Cl–0.005 M β-mercaptoethanol (pH 7.8), and treatment with lysostaphin (50 to 100 μg/ml for 30 to 60 min at 35°C). Staphylococcal cells were suspended in 3 ml of washing buffer and sonicated for 30 s. Cell-free lysates were stored at −70°C for no more than 1 year (2 months for staphylococci) in 1-ml aliquots which were discarded after being thawed and tested.

Reagents. Stock radioisotopes included [1-^{14}C]acetyl coenzyme A (10 μCi/ml; 40 to 60 μCi/μmol; ICN Biomedicals Inc., Irvine, Calif.), [U-^{14}C]ATP (50 μCi/ml; 482 μCi/μmol; Amersham Corp., Arlington Heights, Ill.), and [γ-^{32}P]ATP (10 mCi/ml; 2,900 mCi/μmol; New England Nuclear Corp., Boston, Mass.). With the exception of [γ-^{32}P]ATP, working isotope stocks were prepared and stored as described in Table 1. Owing to the short half-life of ^{32}P, the volume of [γ-^{32}P]ATP necessary to obtain 25 μCi/ml in 0.001 M unlabeled ATP was calculated, and the working stock was freshly prepared on the day of the assay. Substrate AGs were supplied by Bristol Laboratories (amikacin, butirosin, kanamycin, lividomycin, and paromomycin), Schering

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TABLE 1. Modified PBA for AMEs

Component	Step or description	Requirement[a] for indicated assay		
		APH	ANT	AAC
Advance preparation				
Substrates[a]	1. Dilute in distilled H_2O 2. Dispense into microdilution plates, store at −70°C	10 μl (250 μg/ml)	5 μl (1 mg/ml)	10 μl (1 mg/ml)
Lysates	1. Sonic extract (see text) 2. Store at −70°C			
Isotopes	1. Dilute 2. Store at −20°C	[γ-^{32}P]ATP (25 μCi/ml; 0.001 M ATP)	[U-^{14}C]ATP (50 μCi/μmol per ml)	[1-^{14}C]acetyl coenzyme A (5 μCi/μmol per ml)
Buffers[b]	A (0.3 M Tris, 0.3 M maleic acid, 0.03 M $MgCl_2$, 0.01 M dithiothreitol [pH 7.8]) B (0.066 M Tris [pH 7.8], 0.033 M $MgCl_2$, 0.033 M NH_4Cl, 0.0016 M dithiothreitol)	B	A	A
P81 paper	1. Sheets measuring 60 by 120 mm 2. 12 Numbered sections			
Assay				
Isotopes[c]	1. Dilute in buffer 2. Dispense 20 μl into microdilution plate wells	B (1:2)	A (1:4)	A (1:2)
Lysates[c]	Dispense	20 μl	10 μl	20 μl
Reaction	1. Incubate (30 min at 35°C) 2. Return to ice 3. Spot onto P81 paper 4. Air dry (30 s) 5. Stop reaction (1 liter of distilled H_2O, 80°C, 2 min) 6. Wash (three times in 500 ml of distilled H_2O) 7. Dry (e.g., heat lamp)	20 μl	20 μl	25μl
Counting	1. Divide P81 into numbered sections 2. Place into vials containing 7 ml of scintillant 3. Count for 1 min on proper scintillation window	0–670	0–1,000	0–670

[a] See Table 2.
[b] Stored at 4°C.
[c] Maintained on ice.

Corp., Kenilworth, N.J. (gentamicin C, C_1, and C_{1A}; netilmicin, and sisomicin), The Upjohn Co., Kalamazoo, Mich. (neomycin and spectinomycin), Pfizer Inc., New York, N.Y. (streptomycin), and Eli Lilly & Co., Indianapolis, Ind. (tobramycin). Substrates (Table 2) were prepared as shown in Table 1, dispensed into microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) by using a Titertek 12-channel variable-volume micropipette (Flow Laboratories, Inc., McLean, Va.), and stored at −70°C. Phosphocellulose paper (Whatman P81) was obtained from VWR Scientific, San Francisco, Calif.

Enzyme assays. Microdilution plate assays for AMEs were performed as described in Table 1. Isotopes, followed by lysates, were dispensed at the time of the assay into microdilution plates containing substrates. Spotting of reaction mixtures required elevating the phosphocellulose (we used straight pins), using only four equally spaced tips on the multichannel micropipette, and altering the numbering on the paper to correlate with microdilution wells (e.g., wells 1, 4, 7, and 10 on the top one-third of the paper, wells 2, 5, 8, and 11 on the middle one-third, and wells 3, 6, 9, and 12 on the bottom one-third). Conventional AME assays were also performed as described in Table 1, with the following substitutions. Borosilicate glass tubes (10 by 75 mm), rather than microdilution plates, were used for the reactions. All the components, including the substrates, were dispensed on the day of the assay by using a single-channel micropipette (Gilson Pipetman; Rainin Instrument Co., Woburn, Mass.). Reaction mixtures were spotted individually onto pieces of phosphocellulose (numbered and cut to approximately 1 cm^2 before the assay).

Screening assays were performed by the microplate procedure as described in Table 1 except that fewer substrates were tested for each class. Each cell-free lysate was evaluated in a single row (12 wells) of a microplate which contained isotopes, water controls, and the substrates necessary for identifying AMEs to the class level (Table 2).

For all procedures, a substrate was regarded to be modi-

TABLE 2. Substrates used in AME microplate assays

Class	Substrate[a] used in microplate well:											
	1	2	3	4	5	6	7	8	9	10	11	12
AAC	H_2O	K	N	TM	AN	GM	SIS	NET	BUT	PAR	C_1	C_{1A}
ANT	H_2O	K	N	TM	AN	GM	BUT	PAR	S	SPT		
APH	H_2O	K	N	TM	AN	GM	SIS	BUT	LIV	S	SPT	

[a] There were eight identical substrate batteries per 96-well plate. Abbreviations for aminoglycosides: AN, amikacin; BUT, butirosin; GM, gentamicin C; C_1 and C_{1A}, gentamicin C subfractions; K, kanamycin; LIV, lividomycin; N, neomycin; NET, netilmicin; PAR, paromomycin; SIS, sisomicin; SPT, spectinomycin; S, streptomycin; and TM, tobramycin. For screening assays, H_2O, GM, and TM were used for AAC, H_2O, K, N, SPT, and S were used for APH, and H_2O, K, S, and SPT were used for ANT.

fied if radioactive counts were greater than or equal to five times those of water controls (no AG). Subclassification of enzymes was done by using previously described interpretive tables (1, 3, 6, 11).

RESULTS

Tests for subclassifying AMEs (conventional and microplate assays) were performed with all 32 isolates (Table 3). At least one AME was detected in 30 isolates, and 19 produced more than one enzyme. The most common class was ANT, for which five subclasses were detected among 24 isolates, followed by APH (four subclasses among 17 isolates) and AAC (one subclass among 7 isolates). Patterns of modification enabled subclassification of all the enzymes, with the exception of the 3′-APHs detected in five isolates (two isolates of *Acinetobacter calcoaceticus* and one isolate each of "*Salmonella infantis*," *Salmonella typhimurium*, and *Klebsiella pneumoniae*). These enzymes, designated 3′, 5″-APH, could not be further subtyped (e.g., I, II, or III) since butirosin and lividomycin but not amikacin were modified, revealing a pattern not ascribable to a single 3′-APH. Agreement of the results obtained from microplate and conventional assays was complete. For microplate assays, radioactive counts of modified substrates were approx-

TABLE 3. Identification of AMEs by standard and microplate PBAs

Species	Class(es) determined by screening	No. of isolates	Subclass(es) determined by indicated assay[a]					
			AAC		ANT		APH	
			MP	S	MP	S	MP	S
Pseudomonas aeruginosa	ANT, APH	2			2″	2″	3′ (II)	3′ (II)
	ANT, APH	1			3″, 9	3″, 9	3′ (II)	3′ (II)
	AAC, APH	1	6′	6′			3′ (II)	3′ (II)
	APH	1					3′ (II)	3′ (II)
Acinetobacter calcoaceticus	ANT, APH	2			3″, 9	3″, 9	3′, 5″	3′, 5″
	ANT	2			3″, 9	3″, 9		
	No enzyme	2						
Salmonella enteritidis	ANT	6			2″+3″, 9	2″+3″, 9		
"*Salmonella infantis*"	APH	1					3′, 5″	3′, 5″
Salmonella typhimurium	APH	1					3′, 5″	3′, 5″
Klebsiella pneumoniae	APH	1					3′, 5″	3′, 5″
Staphylococcus aureus (methicillin resistant)	ANT	3			4′, 4″	4′, 4″		
	ANT	1			3″	3″		
	APH	1					3′ (IV)	3′ (IV)
	AAC, ANT	1	6′	6′	4′, 4″	4′, 4″		
	ANT, APH	1			6	6	3′ (IV)	3′ (IV)
	AAC, ANT, APH	1	6′	6′	6	6	3′ (IV)	3′ (IV)
	AAC, ANT, APH	1	6′	6′	4′, 4″	4′, 4″	2″	2″
Staphylococcus epidermidis	AAC, ANT, APH	2	6′	6′	4′, 4″	4′, 4″	2″	2″
Staphylococcus hominis	AAC, ANT, APH	1	6′	6′	4′, 4″	4′, 4″	2″+3′ (IV)	2″+3′ (IV)

[a] MP, Microdilution plate PBA; S, standard PBA. Entries refer to the site(s) (carbon) of modification on the AG molecule (e.g., 3′, 5″ means that C-3 on the ′ ring and C-5 on the ″ ring were modified).

TABLE 4. Comparison of costs of performing microplate and conventional assays for APHSs[a]

Assay	Cost (quantity) [avg unit cost] of:											
				Reaction ingredients								
	AG (mg) [7.40/g]	Isotope ([^{32}P]ATP) (μCi) [465.00/ mCi]	Scintillant (ml) [10.00/ liter]	Isotope buffer (ml) [2.91/ liter]	0.001 M ATP (ml) [4.30/g]	Lysate (ml) [31.60/ liter]	Tubes (glass; 10 by 75 mm) (*n*) [23.25/ 1,000]	Microplates (*n*) [65.00/ 100]	Micropipette tips (*n*) [40.00/ 1,000]	P81 paper (cm^2) [5.00/ 2,622 cm^2]	Scintillation vials (*n*) [80.00/ 500]	Labor[b] (h) [10.45/h]
Conventional	0.185 (25.0)	12.79 (27.5)	7.70 (770)	0.003 (1.1)	0.003 (1.1)	0.07 (2.2)	2.53 (110)		5.20 (130)	0.47 (248)	17.60 (110)	33.96 (3.25)
Microplate	0.185 (25.0)	12.79 (27.5)	7.70 (770)	0.003 (1.1)	0.003 (1.1)	0.07 (2.2)		1.30 (2; 110 wells)	9.20 (230)	1.05 (550)	17.60 (110)	15.67 (1.5)

[a] The PBA for APHs tests each of 10 lysates against 10 AGs and one water control. Costs are given in dollars. Total costs: conventional, 80.51; microplate, 65.57.

[b] Federally employed GS-9 laboratory microbiologist.

imately equal to those obtained from conventional assays when expressed as multiples of background counts (water controls).

Microplate screening was performed blindly by using lysates assigned a cryptic number to avoid the identification of isolates. AMEs were correctly identified (to the class level) by this assay when the 32 isolates were tested (Table 3).

A cost comparison for microplate and conventional assays is shown in Table 4. Costs of materials and reagents were obtained from 1985 commercial retail catalogs and in some instances required averaging, owing to variations in prices among suppliers and units of issue. The performance of 10 APH assays by the microplate, as opposed to the conventional, method cost $14.94 less. While the costs of materials and reagents were slightly higher, the performance of microplate assays required substantially less time, with an estimated labor cost savings of $18.29. Two steps in which the greatest time savings were realized included predispensing of substrates and spotting of reaction mixtures, both of which were facilitated by a multichannel micropipette. Once substrate stocks were prepared, 50 or more plates could be prepared in 15 min or less.

DISCUSSION

The convenient identification of mechanisms of resistance is becoming an important component in investigating outbreaks of drug-resistant organisms. Examples of this technology are the chromogenic cephalosporin test for β-lactamase (9) and, more recently, nucleic acid probes specific for genes encoding resistance mechanisms (2, 10, 12). The modified PBA may prove especially useful, since laboratories involved in epidemiologic studies of AG resistance may screen organisms for the presence of AMEs in microdilution plates. Predispensing of substrates and multichannel pipetting further streamline the performance of the assay without sacrificing sensitivity or accuracy.

Unlike β-lactamases, which may be detected without extraction due to secretion or spontaneous autolysis, detection of AMEs continues to require more rigorous lysis procedures. Although not evaluated in the present study, this phase of the assay may also be facilitated, for example, through the use of "mini-preps" which may be obtained from colony suspensions but which nonetheless still require lysis techniques such as osmotic shock or sonication. Another possible variation is that substrate batteries may be altered from those described in this study. These changes are most often related to the availability of various AGs and to the extent of substrate profiling required. The addition of apramycin, for example, may be needed for more in-depth characterization of enzymes that acetylate carbon-3 hydroxyl moieties (5). Screening plates may also be altered so that, in a single microdilution plate, more isolates may be tested against fewer substrates. By using three substrates and one water control in a typical ANT screen, for example, one may evaluate as many as 24 isolates (three different lysates in each of eight rows) in a single plate, but for this class only.

The costs of performing AME assays, of importance primarily within laboratories in which they are routinely performed, would be expected to decrease as more assays are performed, owing to improvements in technical skills and decreases in costs of materials when purchased in bulk quantities. Although cost analyses of AAC and ANT assays were not performed in this study, it can be assumed that savings would approximate those observed for the APH assay.

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